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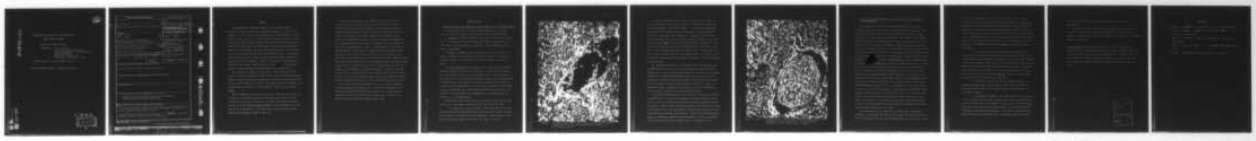

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# CYTOCHEMICAL STUDY RELATED TO LASER APPLICATION

## ANNUAL PROGRESS SUMMARY

Contract No. DAMD 17-74-C4143

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Covering February 1, 1976 to January 30, 1977

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The damage of $CO_2$ laser to cornea of Rhesus monkey has been studied by cytochemical enzyme methods suitable for electron microscopy. NADH diaphorase is used as the marker enzyme for this study.

New $Na^+$-$K^+$ ATPase methods have been developed. It is ~~prepared~~ proposed to study the endothelial damage of cornea by these new methods.

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## SUMMARY

The employment of cytochemical methods with electronmicroscopy is essential to define functional and morphological damage as a result of laser treatment. In the current year, the investigation of NADH diaphorase activity on Rhesus monkey cornea as a viability functional marker has been carried out. Based on this marker study, the damage to the epithelium of the cornea below 20 watts/$cm^2$ for 100 millisec is not detectable. At energy level higher than 65 watts/$cm^2$ for 100 millisec, with the carbon dioxide laser, there is an increase of this enzyme activity observed under the electronmicroscope. This increase may be attributed to the release of enzyme after dissociation of membrane by organelle by the laser treatment. However, it has not been found possible to establish a dose response damage effect at this time because of the limited number of sections available for scanning under the electronmicroscope.

The time effect of lasing was also studied. Longer exposure than 100 millisec at the same watt/$cm^2$ will result in more biological damage than higher energy and shorter exposure, even though such differences are not expected based on total energy absorbed by the tissue. This finding suggests the importance of investigating chronic effect of laser treatment related to the laser safety program.

While the damage to the epithelium of cornea is important, such damage can be repaired. The cellular mechanism of such damage repair process has been investigated in the course of this work. The primary ultrastructural finding with the use of the electroncytochemical methods is the involvement of the wing cells in this repair process. The energy source could very well be the mitochondria in the peripheral region of such cells.

On the other hand, damage to the endothelial layer of the cornea, especially of man, is generally considered to be difficult to repair. Because of the importance of this problem to laser safety, the effort to develop cytochemical probes has been centered on suitable markers for the study of the function of the corneal endothelium. An important enzyme marker for the endothelial function is the $Na^+$-$K^+$ adenosine triphosphatase, more commonly known as transport ATPase, a regulator of ion flow which has been shown by physiologists to relate to corneal hydration. While there are methods reported for the study of this enzyme at the ultrastructural level, preliminary study of these methods showed that they are inadequate for our application. For our purpose, it is necessary that we could correlate the light microscope observation with that of the electronmicroscope, and that the enzyme activity could be observed with cornea fixed in glutaraldehyde so that the morphology of the endothelium is reasonably maintained. Dissatisfied with the current practice, we proceeded to develop alternate methods for the demonstration of this enzyme for electronmicroscopy. Three possible methods were developed to circumvent the deficiency of the current ATPase method (see page 9).

At the time of this progress summary, the use of a newly synthesized 5-nitroindoxyl phosphate ammonium salt as a potassium ion dependent ATPase substrate successfully demonstrated the enzyme activity in rabbit cornea. The extension of this new method to Rhesus monkey cornea should now be a fruitful area of investigation related to laser safety.

PROGRESS REPORT

I. EVALUATION OF $CO_2$ LASER IRRADIANCE ON RHESUS MONKEY CORNEA AT LOW ENERGY LEVEL

In this period, we were mainly concerned with damage at levels below that observable with light microscopy and conventional electronmicroscopy.

a. Materials: Specimens of Rhesus cornea after lasing with carbon dioxide laser at 30 watts/$cm^2$, 20 watts/$cm^2$, and 15 watts/$cm^2$ were evaluated. The exposure time was 100 milliseconds in all samples. The accompanying cornea was used as normal control.

b. Methods: The NADH diaphorase method, based on the use of TNST as a synthetic substrate, has been described in previous reports. This method has also been reported in recent meetings of the Electronmicroscope Society of America.

c. Results and Discussion: Morphology of the cornea under the electron microscope involves two layers in the epithelium: the wing cells facing the tear film and the basal cells facing the Bowman's layer. The Bowman layer is the integral part of the stroma which consists of collagen fibers and keratocytes of elongated shape. The endothelium lies on the opposite end, bordering the stroma via the Descemet membrane, a layer of mainly carbohydrate polyssacharides and some glycoproteins, and a glycocalyx layer facing the aqueous humor. The latter is visible only under the electronmicroscope. Morphologically, the endothelium is a unicellular layer.

Two morphological observations were used as possible damage markers, and based on these, the damage at 30 watts/$cm^2$ was observed with certainty.

1) Damage to the wing cell nucleus. Since these cells are over the basal epithelial cells, and therefore likely bore the initial effect of lasing, this observation is interesting, but not unexpected. Nuclear damage is found, however, only in the center of the lesion. See Figure 1 on page 5.

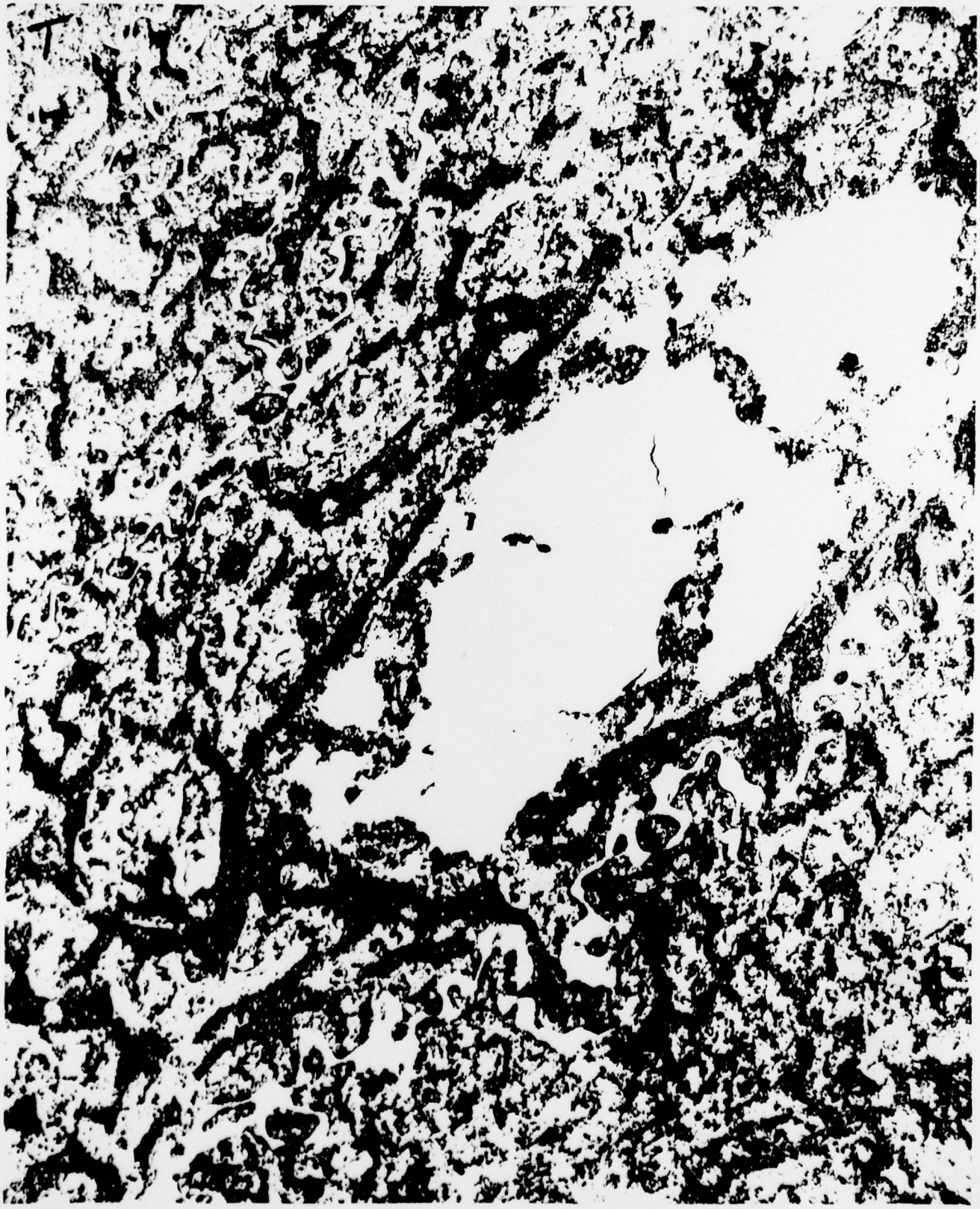

Figure 1. Epithelial damage (Rhesus monkey cornea) after $CO_2$ laser treatment. (30 watts/cm$^2$/100 msec) Note nuclear damage in wing cells. Magnification: 29,000 X

2) Damage to the basal cells. The basal cells may appear normal in their nucleus, yet in their cytoplasm, there was found abnormal thickening of the tonafilaments. See Figure 2 on page 7. Previously, we reported the observation of electrondense deposits attributable to the NADH diaphorase activity in 65 watts/$cm^2$ lasing experiments. Such deposits could be seen also in the 30 watts/$cm^2$ level, and appear as greenish deposits in the thick section of tissue (1μ), as well as described previously. Therefore, we could consider this enzyme activity to have been enhanced by lasing at this energy level. Several attempts were made to use these sections at lower energy level than 30 watts to see if there was a dose response effect. The results were not satisfactory, due mainly to the limitation of the number of sections obtainable in the lesion area.

The endothelium was present in all samples in the 15-30 watts/$cm^2$ range. The NADH diaphorase activity was not visibly altered in endothelium and is perhaps related to the incubation conditions we used. We had used a relatively high substrate concentration (millimolar range) to insure activity for the epithelium. Realizing also that this enzyme is not the only viability marker that we could use, we believe if more samples were possible, a battery of respiratory enzymes, including SDH and cytochromes, could be investigated at the ultrastructural level with the new methods now at hand.

The ultimate threshold safety of the use of laser for man must be defined at cellular level. A 3.2 mm lesion (approximate irradiance diameter) covers about $5 \times 10^6$ cells. Without the aid of both electronmicroscopy and cytochemical probe, it would be difficult to answer questions concerning $CO_2$ laser damage at the cellular level. With such methods, the qualitative observation can be a useful reference for setting threshold safety. With further methodology development in cytochemistry, quantitation with morphology is also an approachable solution to the setting of dose-response range at the biological level.

Figure 2

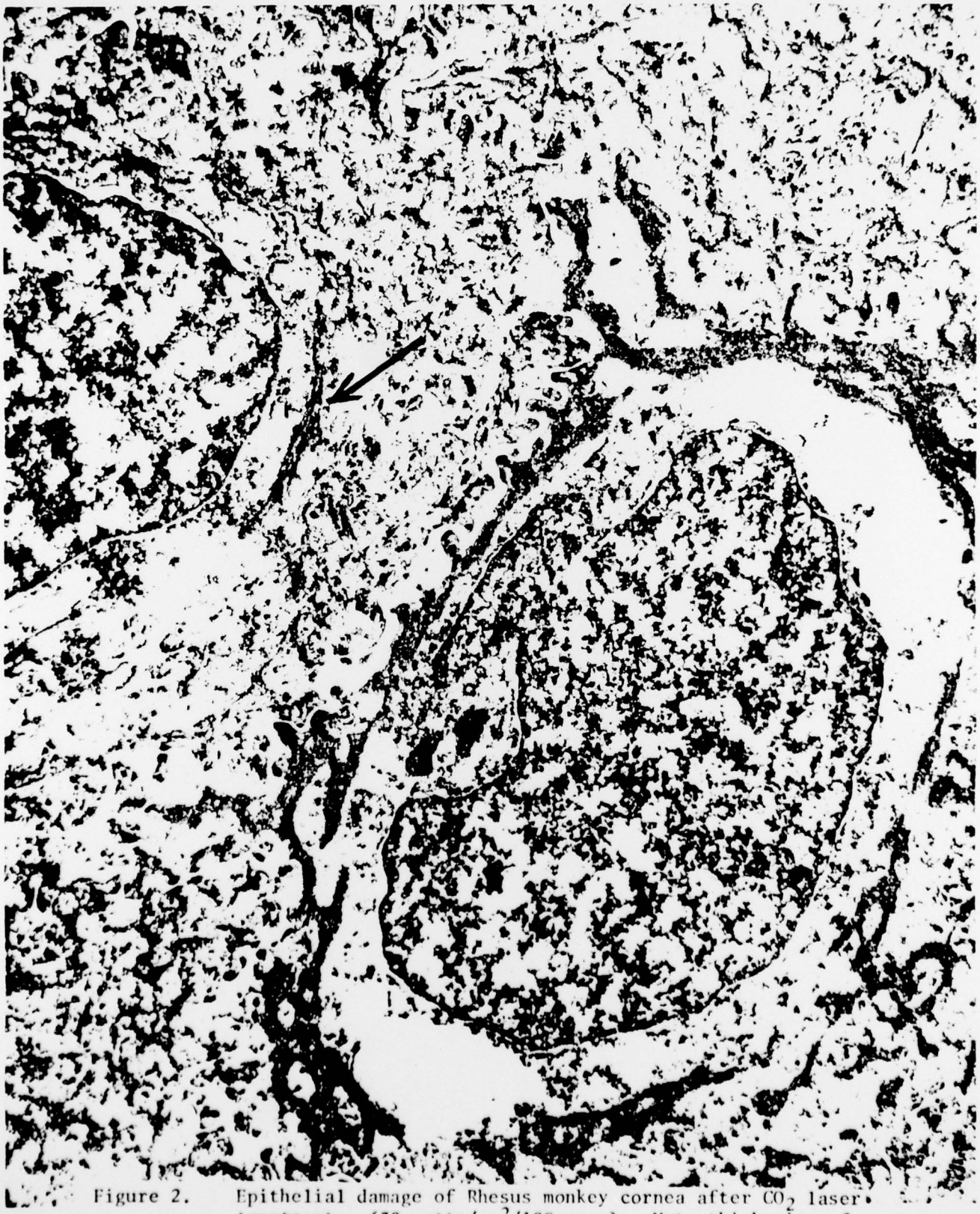

Figure 2. Epithelial damage of Rhesus monkey cornea after $CO_2$ laser treatment. (30 watts/$cm^2$/100 msec) Note thickening of tonafilaments (⟶). Magnification: 22,000 X

## II. $Na^+$-$K^+$ ADENOSINE TRIPHOSPHATASE AS A POTENTIAL CYTOCHEMICAL MARKER FOR CORNEA ENDOTHELIUM

The transparency of cornea is the most important key of vision and is related to the ability of the cornea in controlling water of hydration. This remarkable property of cornea is in turn related to the capacity of cornea endothelium in regulating ion flow and osmosis. The thickness of the Descemet membrane adjacent to the endothelium which consists of polysaccarides, is also affected by the same process of regulation. Furthermore, damage to the endothelial cells in human is not known to repair readily, and thus the understanding of enzymes involved in this process is an important problem in the understanding of corneal opacity and other diseases of the cornea. Largely through the effort of Maurice and other investigators in ophthalmology physiology, $Na^+$-$K^+$ATPase has been implicated as the potential key enzyme for regulating this process. However, no investigation at the electron microscope level has been reported until recently. Leuberger and Novikoff found the presence of enzyme activity which hydrolyzes p-nitrophenyl phosphate in high potassium ion concentration in rat cornea endothelium, but not in epithelium. Since no alkaline phosphatase inhibitor was used in their experiment, the activity they found may reflect both alkaline phosphatase and $Na^+$-$K^+$ ATPase. Nevertheless, this report does suggest the possibility of using this enzyme as a unique marker for endothelium. Unfortunately, formalin was used as fixative in their work, and a great deal of morphology integrity was lost in the electron microscope picture shown in their report. Subsequently, we confirmed their finding that glutaraldehyde inhibits the enzyme activity and therefore other methods that could maintain both morphology and enzyme activity had to be sought.

Three new methods for the demonstration of ATPase have recently been developed in our laboratory and they have been adapted for the demonstration of the $Na^+$-$K^+$ transport ATPase activity in endothelium of the rabbit cornea. While

we are aware that Rhesus monkey cornea should be used as a model for man, the cost is prohibitive for the methodology development stage. Rabbit cornea was therefore used in the evaluation of conditions for the demonstration of this enzyme. An endothelial cell tissue culture provided by the Scheie Eye Institute through the courtesy of Dr. M. Yanoff was also used as testing material. As mentioned earlier, the laser safety study requires methods that could combine light and electron microscope observation for correlation. These new methods have also recognized this latter requirement in the course of their development.

a. Aza-ε-ATP as susbstrate and polynucleotide phosphorylase as coupler to yield a fluorescent polymer poly-aza-ε-adenylic acid (poly-aza-A) as the enzymatic reaction product. This product can react with osmium tetroxide to form an electrondense polymer which is visible under the electron microscope. Potentially this method has the highest sensitivity but requires a good fluorescence microscope for this work. For, if inactivation of enzyme does take place, the fluorescence of the product decreases. However, at the electron microscope level, the stain may still be visible.

b. Aza-ε-ATP as substrate and trap aza-ε-ADP and phosphate as a ternary lead complex which can be viewed with fluorescence microscope, and subsequently reacted with osmium to yield a purplish osmium containing an electrondense product.

c. 5-Nitroindoxyl phosphate as the enzyme substrate, $K^+$ as activator, $Sr^{++}$ as capturing ion, which leads to $SrPO_4$. The precipitated strontium salt can later convert to the lead phosphate. In this reaction, the dinitroindigo formed at the same time from the enzyme reaction, as a result of oxidation of nitroindoxyl, the enzyme reaction product. This method is therefore visible under the light and electron microscope. A preliminary report of this work

has been submitted to the Histochemical Society meeting in April for presentation.

Study with these methods are now being continued, and their evaluation in monkey cornea with the cooperation of Letterman Army Institute of Research promises to provide useful data in the evaluation of the function of endothelium after laser treatment.

Acknowledgement: We acknowledge the advice of Lt. Col. E. Beatrice and Mr. Bruce Stuck of Letterman Army Institute of Research, and their collaborative efforts in providing treated and control cornea for this research project. We also thank Drs. M. Yanoff and N. Colosi of Scheie Eye Institute in providing rabbit corneas and valuable discussions concerning the functional aspect of cornea.

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